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Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: implications for pest management

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Abstract

Wholesale container-grown ornamentals are often maintained at the nursery for at least two growing seasons and are subject to infestation by black vine weevil (BVW), *Otiorhynchus sulcatus* F., for several months each year. Therefore, a potting media amendment aimed at controlling BVW needs to persist for an extended period of time. These studies were conducted to determine the persistence and ecology of *Metarhizium anisopliae* (Metchnikoff) Sorokin incorporated into peat and bark-based potting media with and without a crab meal amendment in container-grown *Picea abies* 'Nidiformis.' Rooted cuttings of *P. abies* were planted into potting media amended with *M. anisopliae* (1 g of formulated product/L; ~6 $\log_{10}$ CFU/g dry potting media). The fungal population in bulk potting media was quantitatively determined using selective media at 14, 21, 28, 35, 49, 63, 77, 91, 105, 119, 143, 175, 203, 231, 258, 287 and 342 days. The fungal population in the rhizosphere was quantitatively determined at 203, 231, 258, 287, and 342 days. *M. anisopliae* colonized the rhizosphere of *P. abies* and the fungal population in the rhizosphere was significantly greater than in the surrounding bulk media. *M. anisopliae* persisted in the peat and bark-based potting media at 6.22 and 5.74 $\log_{10}$ CFU/g dry potting media for 342 days, respectively. Bioassays using bark and peat-based potting media inoculated with *M. anisopliae* at 6 $\log_{10}$ CFU/g dry potting media resulted in 93.5% and 97.5% infection of last instar BVW, respectively. *P. abies* roots inoculated with *M. anisopliae* infected 76% of 2nd–3rd instar BVW. Inoculation of roots with *M. anisopliae* represents a novel method for delivering entomopathogenic fungi and would greatly reduce application costs. Factors associated with fungal biology outside the host may be more important than virulence in a laboratory bioassay.

Keywords: *Metarhizium anisopliae*; *Otiorhynchus sulcatus*; Fungal biology; Rhizosphere colonization

1. Introduction

The inconsistent performance of biological control agents is often associated with an incomplete understanding of the ecological constraints of the biological system in which they are placed. This is particularly true for entomopathogenic fungi. There is little or no knowledge of their biology outside of their insect host. However, these fungi are often inundatively introduced into the environment in the absence of their host in hopes that they will persist and infect their target once the host immigrates into the treated area. One system utilizing such an approach is the use of *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) to control the black vine weevil (BVW), *Otiorhynchus sulcatus* F., (Coleoptera: Curculionidae). The BVW is a polyphagous insect that is a severe pest of field-and container-grown ornamentals as well as small fruit crops worldwide (Moorhouse et al., 1992). Economic losses are mostly associated with poor plant growth due to larval root feeding and lost shipments due to quarantine issues. Other losses are associated with the cost of control, the

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destructive sampling needed for larval scouting and cosmetic quality reduction due to leaf feeding (adult notching of leaves).

Metarhizium anisopliae has been studied extensively for the biological control of a wide range of insect pests, including BVW (Booth and Shanks, 1998; Moorhouse et al., 1993a,b; Poprawski et al., 1985; Soares et al., 1983) and various other soil borne pests such as *Popillia japonica* (Coleoptera: Scarabaeidae) (Villani et al., 1994), *Ligyrus subtropicus* (Coleoptera: Scarabaeidae) (Raid and Cherry, 1992), *Antitrogus parvulus* (Coleoptera: Scarabaeidae) (Samuels et al., 1990), *Adoryphorus couloni* (Coleoptera: Scarabaeidae) (Rath, 1988) and *Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae) (Krueger and Roberts, 1997). The use, however, of *M. anisopliae* and other entomopathogenic fungi for control of soil borne insects is often inconsistent due to limited spore redistribution and persistence (Storey and Gardner, 1987, 1988; Storey et al., 1989).

Increased understanding of the ecology of entomopathogens outside their host has enhanced our ability to effectively utilize entomopathogens as biological control agents. For example, Lewis and colleagues (Bing and Lewis, 1991, 1992) observed that *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) grows endophytically within the green tissues of *Zea mays* L. (Cyperales: Poaceae). They then demonstrated that endophyte forming isolates of *B. bassiana* effectively managed populations of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae) (Lewis et al., 2002) while being non-pathogenic to *Z. mays* (Lewis et al., 2001).

Isolates of entomopathogenic fungi traditionally have been selected for development as biological control agents based on bioassay results from the laboratory with little emphasis on understanding other fungal traits. Recently, an isolate of *M. anisopliae* (ARSEF 1080) was demonstrated to be rhizosphere competent (Hu and St. Leger, 2002). While the notion of fungal populations increasing in the rhizosphere is not new, this was the first report of an entomopathogenic fungus doing so. In field studies, the population of *M. anisopliae* in bulk soil decreased from 10^5 to 10^3 propagules/g soil after several months, while populations in the inner rhizosphere of cabbage plants remained at 10^5 propagules/g soil (Hu and St. Leger, 2002). A significantly larger fungal population in the inner rhizosphere compared to the outer rhizosphere suggests that response to root exudates is involved in the rhizosphere effect (Hu and St. Leger, 2002) or that sporulation is enhanced in the rhizosphere. Root-deposited photosynthate is an important source of readily available carbon for microbes in the rhizosphere (Butler et al., 2003). Rhizosphere competence is an attribute of a microbe but the rate of colonization also depends on the traits of the host (O'Connell et al., 1996).

Biological control agents differ fundamentally from chemical agents in that in order to be effective, they must proliferate in the environment they are introduced (Nelson et al., 1994). Proliferation of rhizosphere competent microbes for the biological control of soil borne plant diseases has been demonstrated using fungi (Harman, 1992) and bacteria (Kloepper and Beauchamp, 1992). For consistent use of microbes for biological control of plant diseases, it is essential that the biology and ecology of the biological control agent be understood completely (Lo et al., 1998). An understanding of the parameters impacting the ability of *M. anisopliae* to proliferate, maintain an efficacious population and persist in the microenvironment where control is desired would improve biological control efficacy.

All filamentous fungi with chitin as a major cell wall component produce chitinases at all periods of active growth (Gooday et al., 1992). Several insect pathogens including *M. anisopliae*, *M. flavoviride* Gams and Rozsypal (Hypocreales: Clavicipitaceae) and *B. bassiana* produce a complex mixture of chitinolytic enzymes during growth on insect cuticle (St. Leger et al., 1996). The production of chitinase by *M. anisopliae* is dependent on chitin availability (de Siqueria Pinto et al., 1997; St. Leger et al., 1996). *M. anisopliae* produces a heterogenous array of chitinases which may play a role in its ability to adapt to different environments (St. Leger et al., 1993).

The objectives of these studies were to quantify the population of *M. anisopliae* over time in peat and bark-based potting media, determine the effect of crab meal amendment (source of chitin) on the population size and persistence of *M. anisopliae*, determine if *M. anisopliae* colonized the rhizosphere of *Picea abies* (L.) Karst. (Pinales: Pinaceae) 'Nidiformis' and determine if roots inoculated with *M. anisopliae* were able to protect plants from BVW larval feeding.

2. Materials and methods

Two types of soilless potting media typically used in container grown nursery production were used in the experiments. A 2:1 mixture of peat moss (Sunshine Mix #3, Sun Gro Horticulture, Bellevue, WA) and turkey grit (Cherry Stone Grit #3, New Ulm, MN) and a bark-based media (OBC Northwest Nursery Mix #1, OBC Northwest, Canby, OR) which consists of 70% fine bark, 20% mulch, 10% pumice. A commercial formulation of *M. anisopliae* (strain F52) [Earth BioSciences (New Haven, CT [formally Taensa Company])] was used. The formulated product consisted of *M. anisopliae* that had sporulated on rice grains and was then dried. The crab meal amendment used was Eco-Logic (AgriGulf, Bayou La Batre, AL).

The experiment was arranged as a randomized complete block in a split–split plot design with four replications. The whole plot was the type of soilless potting media (peat, bark), the sub-plot the *M. anisopliae*

application (control, incorporated granules, premix) incorporated at the rate of 1 g of formulated product/L of potting media and the sub–sub plot the rate of crab meal amendment (0, 0.68 or 1.36 kg/yd^3 of potting media).

2.1. Media preparation

All experimental treatments were prepared by mixing the appropriate ingredients in a 4 L twin-shell blender (Patterson-Kelley, East Stroudsburg, PA). Each treatment was mixed for 7 min to ensure that the fungus and/or crab meal amendment were incorporated uniformly.

Seven days prior to planting, the premix treatment was prepared by incorporating the full allotment of *M. anisopliae* and crab meal into one-third the total volume of potting media required for each treatment. These concentrated mixtures (premix) were allowed to incubate in plastic pails covered with aluminum foil in the greenhouse head house for 7 days. Ambient temperatures in the head house were approximately 24–28 °C. After 7 days, the premix was mixed with the remaining potting media for each respective treatment. The premix procedure was performed to provide *M. anisopliae* the chance to germinate and begin to grow, thus potentially increasing the overall inoculum level in the potting media.

Rooted cuttings of *P. abies* 'Nidiformis' were potted with the media from each treatment into $8.9 \times 8.9 \times 8.9$ cm pots (McConkey, Wilsonville, OR) and maintained in a screenhouse where they were exposed to ambient conditions. Each treatment consisted of 17 plants, one of which was selected randomly and destructively sampled on each sample date.

2.2. Bulk media processing

Bulk media were sampled at 14, 21, 28, 35, 49, 63, 77, 91, 105, 119, 143, 175, 203, 231, 258, 287, and 342 days after potting. The monthly maximum, minimum and mean temperatures, respectively, for samples collected days 14, 21, 28, and 35 (28.3, 9.7, and 19.1 °C), days 49 and 63 (26.6, 8.3, and 17.2 °C), days 77 and 91 (18.7, 4.1, and 11.4 °C), days 105 and 119 (12.9, 2.3, and 7.6 °C), day 143 (9.7, 2.8, and 6.2 °C), day 175 (10.9, 2.8, and 6.9 °C), day 203 (11.5, 1.5, and 6.5 °C), day 231 (13.6, 5.2, and 9.4 °C), day 258 (14.4, 4.9, and 9.6 °C), day 287 (19.5, 6.5, and 13.0 °C), and 342 (25.5, 9.5, and 17.5 °C). Due to the large number of samples, two replications were sampled and processed completely on subsequent days. Plants were removed from the potting media by grasping the top of the plant and gently pulling as the contents of the pot were dumped out and set aside. Ten grams of bulk potting media were placed in a plastic 250 ml Erlemyer flask containing 90 ml of 0.05% Tween 80 solution, shaken (250 rpm) for 20 min at room temperature, then placed in an ultrasonic cleaner (Model 5210, Branson Ultrasonic, Danbury, CT) for 2 min. Serial dilutions were plated using a spiral plater (iUL Instruments, Barcelona, Spain) onto two plates of media selective for *M. anisopliae* (Veen and Ferron, 1966). Plates were incubated in complete darkness at 28 °C for 4 days. The number of CFU/g dry bulk media was averaged across replicate plates for each sample. To ensure that the colonies counted were *M. anisopliae*, a total of 20 colonies morphologically identical to those counted as *M. anisopliae* were randomly selected from a number of different plates on each sample date and aseptically transferred to potato dextrose agar (PDA) and allowed to sporulate. The colonies transferred were identified based on macro and microscopic characteristics (Humber, 1997). All colonies transferred to PDA throughout the study were *M. anisopliae*.

2.3. Rhizosphere processing

Based upon the report of *M. anisopliae* as a rhizosphere competent organism (Hu and St. Leger, 2002), the ability of F52 to colonize the rhizosphere of *P. abies* was assessed beginning with the sample collected on day 203. In the context of these studies, I used the definition of rhizosphere competence proposed by Schmidt (1979) who defined "rhizosphere competent" microorganisms as those that show enhanced growth in response to developing roots or a classical rhizosphere effect. The rhizosphere population (CFU/g dry rhizosphere media) was determined from treatments of both potting media types, *M. anisopliae* applications and amendments of 0 and 0.68 kg crab meal/yd^3. To quantify the fungal population in the rhizosphere, harvested plants (see above) were shaken gently until only media tightly adhering to the root remained (rhizosphere). Plants were cut at their bases and the above ground portion discarded. The roots were placed into a sterile plastic bag (Nasco, Modesto, CA) and held at 4 °C until processed (2–3 days). *M. anisopliae* does not germinate or grow at temperatures at or below 4 °C (Ekesi et al., 1999; Hallsworth and Magan, 1999). To quantify the *M. anisopliae* population in the rhizosphere, the entire root system from each plant was placed into a plastic 250 ml Erlemyer flask containing 90 ml of 0.05% Tween 80 solution and processed as above. To quantify the amount of rhizosphere media on the root system of each plant sampled, the suspension remaining in each flask (once the roots were removed) was poured into a pre-weighed aluminum pan. Each flask was carefully flushed with distilled water to remove all soil particles. Pans containing the suspension were placed in a 38 °C drying oven until dry (approximately 24 h) and weighed.

2.4. Larval bioassay

A laboratory bioassay was performed at the conclusion of the above described experiment with the peat and bark-based media without a crab meal amendment.

Potting media of each type were prepared (as described previously) with a target concentration of 1×10^6 spores of *M. anisopliae* (strain F52)/g dry potting media. The purpose of the bioassay was to determine if the mean fungal inoculum observed in both potting media for the duration of the above experiment was adequate to infect last instar BVW. The peat and bark-based media were inoculated with grains of rice on which the *M. anisopliae* had been allowed to sporulate. Each treatment was mixed in 4 L twin-shell blenders as described previously. To verify the inoculation rate, samples of each treatment were immediately taken and the CFU/g dry potting media determined as described previously. The mean spore concentration in the bioassay was 1.87×10^6 CFU/g dry potting media (determined via spiral plater). Approximately 200 cm^3 of the inoculated potting media was placed in 236 cm^3 plastic containers along with 20 last-instar BVW and a carrot slice as a food source. The cups were incubated in complete darkness at 24 °C and larval mortality recorded at 14 days. Larvae were obtained from a laboratory colony maintained at the USDA-ARS, Horticultural Crops Research Laboratory, Corvallis, OR. The experiment was arranged in a randomized complete block design, replicated four times and included an untreated control for both potting media types. The experiment was performed twice.

2.5. Root inoculation

Rooted cuttings of *P. abies* 'Nidiformis' were dipped into 400 ml of a 0.05% Tween 80 solution containing 2×10^6 spores/ml of *M. anisopliae* (strain F52), placed on sterile paper towel on the laboratory bench top and allowed to dry. The roots of the cuttings were potted with the 2:1 mixture of peat moss and turkey grit (described above) and placed in petri dishes (100×30 mm) with a small opening for the plant stem. The petri dishes served as small rhizotrons which allowed for the observation of larvae feeding on the roots as well as any larvae that became infected with *M. anisopliae*. The plants were maintained in an upright position in the greenhouse (27 ± 3 °C) for 5 days and watered as needed. After 5 days, five 2nd–3rd instar BVW larvae from the laboratory colony were added to each dish and the plants returned to the greenhouse. The experiment was performed in a completely randomized design with six replications. Control plants were dipped into 400 ml of 0.05% Tween 80 solution only and BVW larvae added in the same manner. The experiment was performed twice with individual batches of spore and control suspensions. Thirteen days after larvae were placed in the petri dishes, larvae were observed through the lid and base of the petri dish on treated plant roots infected with *M. anisopliae*. At this point, the plants were returned to the laboratory and the contents of each dish carefully searched for infected or healthy BVW larvae.

2.6. Data analysis

Analysis of data from the bulk media sampling was performed using the General Linear Models Procedure (GLM) with Tukey's multiple range test used to separate means (SAS Institute, 1999). The data from each of the 17 sampling dates were analyzed separately. *M. anisopliae* was never isolated from the control treatment and these data were removed when calculating the mean fungal population ($\log_{10}$ CFU/g dry potting media). The data were analyzed as a split–split plot design with hypothesis testing used to pair the correct error terms in the *F* test. Although samples were collected from each treatment over time, a repeated measures analysis was not required because plants were maintained individually, destructively sampled and the fungal population from each pot quantified only once.

A one sample *t* test was performed to determine if the difference between the $\log_{10}$ CFU/g dry potting media in the rhizosphere and bulk media of each plant sampled was significantly different from zero (SAS Institute, 1999). The *t* test revealed that on each sample date, the difference between the mean $\log_{10}$ CFU/g dry soil in the rhizosphere and bulk media was significantly greater than zero. These difference data were then analyzed using GLM and Tukey's multiple range test used to separate means (SAS Institute, 1999).

Data from the larval bioassays and root inoculation studies were analyzed using GLM and *t* test used to separate means (SAS Institute, 1999). A test of homogeneity of variance was performed to detect variation between the two runs of each experiment (Little and Hills, 1978). Variability was not significant between runs of either experiment and the data from the runs were combined for analysis.

3. Results

3.1. Fungal persistence

Regression analysis was used to describe the relationship between the *M. anisopliae* population ($\log_{10}$ CFU/g dry potting media) and sample date in both potting media. Throughout the course of the study there was a significant decline in the fungal population in both the peat-based ($\log_{10}\text{CFU} = 6.69 - 0.0016\,d$; $R^2 = 0.83$) and bark-based potting media ($\log_{10}$ CFU $= 6.63 - 0.0036\,d$; $R^2 = 0.80$). The decline in fungal density in both media types was very gradual, particularly in the peat-based potting media. There were no significant differences in fungal density between media types for the first 119 days post-treatment (Fig. 1). The fungal density in both types of potting media nearly mirrored one another the first 119 days post-application with no significant difference in fungal population between the two potting media.

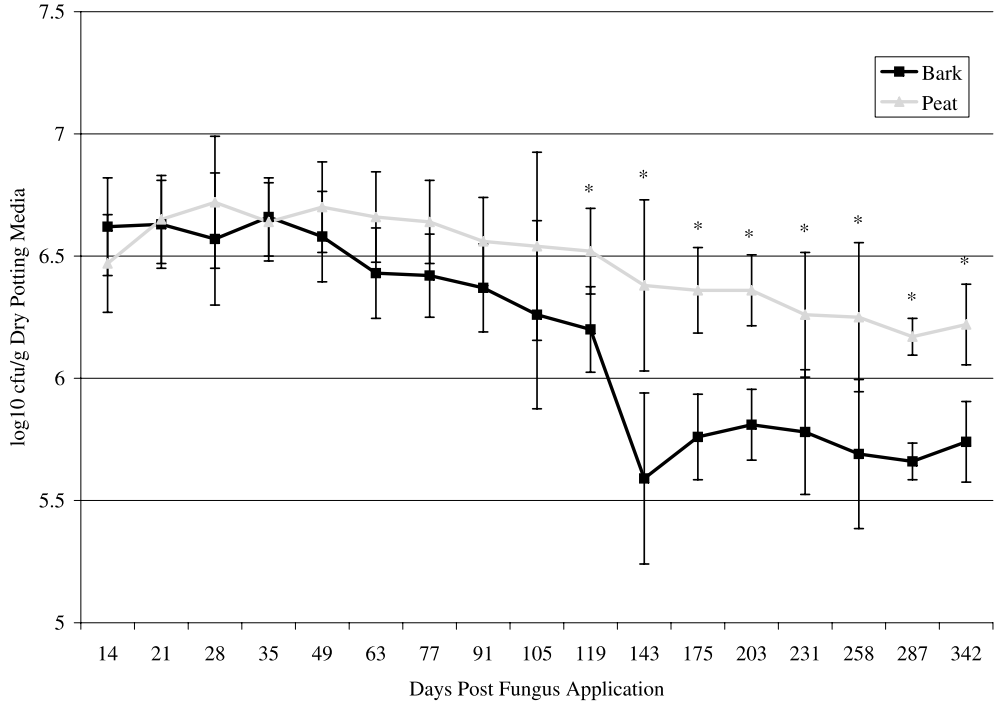

Fig. 1. Mean $\log_{10}$ CFU/g of dry potting media (±SD) (with the control treatments removed from the analysis) of *Metarhizium anisopliae* (strain F52) in peat and bark-based potting media for 342 days. Points denoted with an (*) are significantly different ($P < 0.05$).

Following the marked decline in fungal density in the bark-based media on day 143, the fungal density in the peat-based media was significantly higher on each of the subsequent sample dates (Fig. 1).

While the fungal inoculum load in the potting media was not immediately quantified after fungal incorporation at the beginning of the persistence study, there appears to have been an initial increase in the fungal population in the premix treatment (as hypothesized) that was sustained throughout the course of the experiment. The fungal population ($\log_{10}$ CFU/g dry potting media) appears to have increased significantly during the 7-day incubation period as indicated by the significantly higher fungal population in the premix treatment on day 14 (Fig. 2). When the aluminum foil covers were removed from the top of the pails containing the concentrated fungal mixture, *M. anisopliae* hyphal growth was visibly evident in the potting media. The fungal population in both treatments followed a similar pattern, however, the population in the premix treatment was significantly higher (approximately $0.5 \log_{10}$ CFU/g dry potting media) throughout the course of the experiment (Fig. 2). Regression analysis was used to describe the relationship between the *M. anisopliae* population ($\log_{10}$ CFU/g dry potting media) and sample date for both means of fungal application. There were a significant declines in the fungal population in both the incorporated ($\log_{10}$ CFU $= 6.43 - 0.0029\,d$; $R^2 = 0.87$) and premix ($\log_{10}$ CFU $= 6.90 - 0.0023\,d$; $R^2 = 0.77$) applications. The declines in fungal population utilizing both types of fungal application were very gradual. While the rate of decline in fungal population was very similar in the incorporated and premix applications, the fungal population in the premix application was significantly higher initially and this difference was maintained over the 342 days of the study.

3.2. Larval control

Incorporation of 1.87×10^6 CFU/g dry potting media resulted in significant levels of larval infection ($P < 0.001$). The levels of last instar BVW infection with *M. anisopliae* in the bark and peat-based potting media were 93.5 and 97.5%, respectively.

3.3. Rhizosphere competence

The one sample *t* test revealed that on each sample date the difference between the *M. anisopliae* population in the rhizosphere and surrounding bulk media was significantly greater than zero (Table 1). These data indicate that not only did *M. anisopliae* colonize the rhizosphere of *P. abies*, but the *M. anisopliae* population responded favorably to the rhizosphere microclimate. The mean difference in *M. anisopliae* population in the rhizosphere ranged from 0.65 to $1.28 \log_{10}$ CFU/g media. The GLM analysis of the data consisting of the mean difference between the rhizosphere and bulk media population from each plant sampled showed that potting media type was the only parameter studied that had any

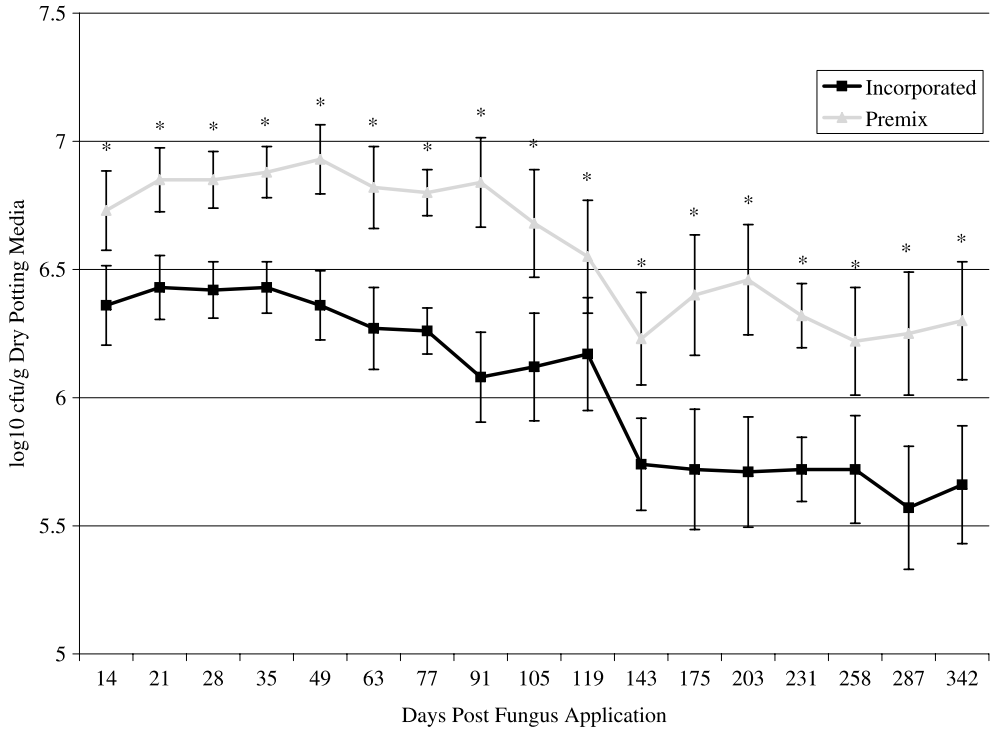

Fig. 2. Mean $\log_{10}$ CFU/g of dry potting media (±SD) of *Metarhizium anisopliae* (strain F52) averaged over peat and bark-based potting media in each of the fungal incorporation treatments (with the control treatments removed from the analysis). Incorporated—1 g of formulated fungal granules/L of potting media; Premix—final concentration of 1 g of formulated fungal granules/L of potting media. A premix (a third of the total volume of potting media for each treatment inoculated with the total amount of fungal granules) were allowed to incubate in plastic pails in the greenhouse head house for 7 days covered with aluminum foil. This premix was added to the remaining volume of potting media for each respective treatment. Points denoted with an (*) are significantly different ($P < 0.05$).

Table 1
Mean difference (±SD) between the rhizosphere and bulk media *Metarhizium anisopliae* populations ($\log_{10}$ CFU/g dry potting media) and the probability that the observed difference was greater than zero

Day [a]	Mean difference	*t* statistic [b]	*df*	$P > t$
203	0.65 ± 0.48	10.73	30 [c]	<0.001
231	0.71 ± 0.64	8.76	31	<0.001
258	0.83 ± 0.32	19.47	31	<0.001
287	1.28 ± 0.48	22.26	31	<0.001
342	0.97 ± 0.32	23.48	31	<0.001

[a] Number of days post-fungal application that samples were taken.
[b] A one sample *t* test performed to determine if the difference between the $\log_{10}$ CFU/g dry potting media in the rhizosphere and bulk media of each plant sampled was significantly different than zero (SAS Institute, 1999).
[c] One less degree of freedom than expected due to contaminated plates from one sample.

significant effect on the size of the difference observed. The difference in *M. anisopliae* population between the rhizosphere and bulk media was greatest in the peat-based potting media on three of the five sample dates (Table 2). There were no significant differences in the mean difference between the rhizosphere and bulk media *M. anisopliae* populations due to fungal application or crab meal amendment, nor were there any significant interactions.

3.4. Root inoculation

Inoculation of roots of *P. abies* with 2×10^6 spores/ml of *M. anisopliae* was a successful fungal delivery system. A significant percentage (±SEM) (76 ± 9%) of the BVW larvae feeding on the fungal-treated roots were infected with *M. anisopliae* ($P < 0.001$). There were no larvae infected with *M. anisopliae* in the control treatment. All of the infected larvae were located near the root. The roots of the plants where infected larvae were found were visually inspected for signs of larval feeding. Initial larval feeding was often observed where larvae had located plant roots and begun to feed before succumbing to infection. These data demonstrate that inoculation of plant roots with a rhizosphere competent isolate of *M. anisopliae* is a useful, feasible approach for managing BVW and perhaps other root-feeding insects.

4. Discussion

The ability of *M. anisopliae*-treated roots to control BVW larvae indicates the potential to use colonized roots as a delivery system for fungal biological control agents of root-feeding insects. There is a large volume of published work in the field of plant pathology on the use of

Table 2
Mean difference and 95% confidence interval between the rhizosphere and bulk media *Metarhizium anisopliae* populations (log_{10} CFU/g dry potting media) due to potting media type

Day [a]	Potting media	Mean difference [b]	Lower[c]	Upper [d]
203	Bark	0.44a	0.09	0.78
	Peat	0.82a	0.67	0.97
231	Bark	0.38a	0.17	0.58
	Peat	1.04b	0.83	1.24
258	Bark	0.69a	0.55	0.83
	Peat	0.97b	0.83	1.11
287	Bark	1.14a	0.90	1.38
	Peat	1.43a	1.19	1.67
342	Bark	0.87a	0.76	0.98
	Peat	1.08b	0.97	1.19

[a] Number of days post-fungal application that samples were taken.
[b] GLM used to determine if the difference between the log_{10} CFU/g dry potting media in the rhizosphere and bulk media of each plant sampled was significantly different on each individual sample date. Means separated with Tukey's multiple range test (SAS Institute, 1999).
[c] Lower limit of the 95% confidence interval of the mean (SAS Institute, 1999).
[d] Upper limit of the 95% confidence interval of the mean (SAS Institute, 1999).

rhizosphere competent organisms (both bacteria and fungi) for the microbial control of plant diseases (Harman, 1992; Kloepper and Beauchamp, 1992). This provides a wealth of knowledge which can be used to develop the use of rhizosphere competent entomopathogenic fungi for pest control. When selecting an isolate, understanding factors associated with entomopathogen biology outside their insect host may be more important than its virulence in a laboratory bioassay of insect infection.

Currently, when protecting plants from root-feeding insects using entomopathogenic fungi, efforts are concentrated on applying large amounts of inoculum to increase the fungal population throughout the bulk soil. This technique presents numerous problems including: (1) a requirement for large quantities of fungal inoculum, often making applications uneconomical, (2) it is difficult to get the fungal propagules applied to the soil surface to penetrate into the soil more than a few centimeters and (3) a large amount of time, money and effort is spent protecting areas of the bulk soil where the pest either does not occur or is not of concern. By developing techniques to use the roots as a delivery system for entomopathogenic fungi, the costs and logistics of biological control would be much more favorable. As demonstrated here, it is possible to use roots treated with levels of entomopathogenic fungal inoculum comparable to those colonizing the rhizosphere to infect insects and protect the plant from root feeding. Regardless of the root-feeding insect considered, they all must feed on plant roots in order to complete their development and thus come into contact with the rhizosphere competent fungus and become infected. By using this approach, the application of entomopathogenic fungi should be economical as only the roots of small plants (rooted cuttings, tissue culture) or potentially seeds would have to be inoculated. If rhizosphere competent isolates of entomopathogenic fungi actively colonize elongating roots and persist with the growing plant, plants could be protected for extended periods of time.

In these studies were the *M. anisopliae* rhizosphere population was quantified, plants were grown in potting media in which *M. anisopliae* was uniformly incorporated. Therefore, the entire root system was in contact with an inoculum source throughout the length of the study. The ability of *M. anisopliae* to colonize elongating roots is unclear. *M. anisopliae* was isolated at a soil depth of 10 cm from plants in which the inoculum was applied as spore suspension on the soil surface, suggesting some degree of vertical movement either through colonization or via percolating water (Hu and St. Leger, 2002). The largest population of *M. anisopliae* in the inner and outer rhizosphere was observed in the first 2 cm of root and declined significantly from 2 to 10 cm (Hu and St. Leger, 2002). Most of the *M. anisopliae* inoculom on cabbage roots was easily removed by washing which suggests weak adhesion to the root (Hu and St. Leger, 2002) or that spores are easily removed.

Therefore, the criteria for defining a rhizosphere competent entomopathogenic fungus will have to evolve to keep pace with the goals and methodology in mind when developing them. The key concept of root colonization by beneficial bacteria is that the bacteria grow in the presence of natural flora (Schroth and Hancock, 1982) and therefore are competitive with bacteria and fungi present in the soil. More recently the definition of "rhizosphere competence" has been refined when considering biological control agents to, "the ability of a microorganism, applied by seed treatment, to colonize the rhizosphere of developing roots" (Baker, 1991). A definition for rhizosphere competent entomopathogenic fungi would require that the fungus grow and persist in the presence of natural flora in the soil or potting media and have the ability to colonize the rhizosphere around developing roots at populations great enough to infect pest insects feeding on the root.

Metarhizium anisopliae was also shown to persist well in both peat and bark-based bulk potting media for up to 342 days. The ability of *M. anisopliae* to persist in horticultural potting media (often microbially impoverished because of the acidic peat content) may not translate well in microbially diverse field soils. The cause for the marked reduction in fungal density in the bark-based media on day 143 is unclear. Moisture retention might be one of the most significant factors affecting microbial activity (Griffin, 1969, 1981). Soil temperature also can impact the survival and infectivity of *M. anisopliae* in

soil (Ekesi et al., 2003; Li and Holdom, 1993). Fungal persistence generally is reduced in soils with higher water contents. The samples collected on days 119 and 143 correspond to November 18th and December 12th, 2003, respectively. Ambient winter conditions in Oregon are cool and damp which may have allowed the bark-based potting media to remain wet for a prolonged period. Decline of *M. anisopliae* in wet soils (0 and −2.0 kPa) occurs at 30 and 60 days in clay and sandy loam soil, respectively (Li and Holdom, 1993).

The results of the larval bioassays indicate that the mean level of inoculum (~6 $\log_{10}$ CFU/g dry potting media) observed in both potting media types over the duration of the experiment was sufficient to provide excellent control of last instar BVW. This indicates that the traditional approach of inundating the system (potting media) with propagules of *M. anisopliae* would provide growers with nearly a year of residual control in covered production areas. The next step in developing *M. anisopliae* for use in container-grown ornamentals is to determine fungal persistence in containers located outdoors in the nursery.

The use of a premix treatment would also provide benefits to growers wishing to employ *M. anisopliae* in their BVW control program. This type of program would be particularly useful for plants with roots that are not colonized by *M. anisopliae* or for those plants which rhizosphere colonization had not been demonstrated. The amount of fungal product required to obtain BVW control when employing a premix treatment is less than required when incorporating the fungus directly into the potting media at planting. Substantially less fungus would have to be used when utilizing a premix application to end up with the same inoculation rate ($\log_{10}$ CFU/g dry potting media). If this phenomenon could be understood further and manipulated to be even more dramatic, it could potentially be used with other fungal biological control agents to increase inoculum while decreasing the cost to the user. Such an approach could make the traditional application of fungal biological agents more competitive with chemical insecticides.

Our traditional approaches to the biological control of soil borne pests with entomopathogenic fungi historically have not been very successful, and when success has been demonstrated, it has not been consistent. A completely new approach which shifts the focus of our efforts away from laboratory bioassay data and onto fungal ecology may very well lead to success.

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References

Baker, R., 1991. Induction of rhizosphere competence in the biological control fungus *Trichoderma*. In: Keister, D.L., Cregan, P.B. (Eds.), The Rhizosphere and Plant Growth. Kluwer Academic Publishers, Dordrecht, Boston and London, pp. 221–228.

Bing, L.A., Lewis, L.C., 1991. Suppression of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) by endophytic *Beauveria bassiana* (Balsamo) Vuillemin. Environ. Entomol. 20, 1207–1211.

Bing, L.A., Lewis, L.C., 1992. Endophytic *Beauveria bassiana* (Balsamo) Vuillemin in corn: the influence of the plant growth stage and *Ostrinia nubilalis* (Hübner). Biocontrol Sci. Technol. 2, 39–47.

Booth, S.R., Shanks Jr., C.H., 1998. Potential of a dried rice/mycelium formulation of entomopathogenic fungi to suppress subterranean pests in small fruits. Biocontrol Sci. Technol. 8, 197–206.

Butler, J.L., Williams, M.A., Bottomley, P.J., Myrold, D.D., 2003. Microbial community dynamics associated with rhizosphere carbon flow. Appl. Environ. Microbiol. 69, 6793–6800.

de Siqueria Pinto, A., Barreto, C.C., Schrank, A.C., Ulhoa, J., Vainstein, M.H., 1997. Purification and characterization of an extracellular chitinase from the entomopathogen *Metarhizium anisopliae*. Can. J. Microbiol. 43, 322–327.

Ekesi, S., Maniania, N.K., Ampong-Nyarko, K., 1999. Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti.*. Biocontrol Sci. Technol. 9, 177–185.

Ekesi, S., Maniania, N.K., Lux, S.A., 2003. Effect of soil temperature and moisture on survival and infectivity of *Metarhizium anisopliae* to four tephritid fruit fly puparia. J. Invertebr. Pathol. 83, 157–167.

Gooday, G.W., Zhu, W.-Y., O'Donnell, R.W., 1992. What are the roles of chitinases in the growing fungus. FEMS Microbiol. Lett. 100, 387–392.

Griffin, D.M., 1969. Soil water in the ecology of fungi. Annu. Rev. Phytopathol. 7, 289–310.

Griffin, D.M., 1981. Water potential as a selective factor in the microbial ecology of soils. In: Parr, J.F., Gardner, W.R., Elliot, L.F. (Eds.), Water Potential Relations in Soil Microbiology. Special Publication No. 9, Soil Sci. Soc. Am., Madison, WI, pp. 141–151.

Hallsworth, J.E., Magan, N., 1999. Water and temperature relations of growth of the entomogenous fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus*. J. Invertebr. Pathol. 74, 261–266.

Harman, G.E., 1992. Development and benefits of rhizosphere competent fungi for biological control of plant pathogens. J. Plant Nutr. 15, 835–843.

Hu, G., St. Leger, R., 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. Appl. Environ. Microbiol. 68, 6383–6387.

Humber, R.A., 1997. Fungi: identification. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA, pp. 153–185.

Kloepper, J.W., Beauchamp, C.J., 1992. A review of issues related to measuring colonization of plant roots by bacteria. Can. J. Microbiol. 38, 1219–1232.

Krueger, S.R., Roberts, D.W., 1997. Soil treatment with entomopathogenic fungi for corn rootworm (*Diabrotica* spp.) larval control. Biol. Control 9, 67–74.

Lewis, L.C., Bruck, D.J., Gunnarson, R.D., Bidne, K.G., 2001. Assessment of plant pathogenicity of endophytic *Beauveria bassiana* in Bt transgenic and non-transgenic corn. Crop Sci. 41, 1395–1400.

Lewis, L.C., Bruck, D.J., Gunnarson, R.D., 2002. On-farm evaluation of *Beauveria bassiana* for control of *Ostrinia nubilalis* in Iowa, USA. BioControl 47, 167–176.

Li, D.P., Holdom, D.G., 1993. Effect of soil matric potential on sporulation and conidial survival of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes). J. Invertebr. Pathol. 62, 273–277.

Little, T.M., Hills, F.J., 1978. Agricultural Experimentation: Design and Analysis. Wiley, New York.

Lo, C.-T., Nelson, E.B., Hayes, C.K., Harman, G.E., 1998. Ecological studies of transformed *Trichoderma harzianum* Strain 1295-22 in the rhizosphere and on the phylloplane of creeping bentgrass. Phytopathology 88, 129–136.

Moorhouse, E.R., Charnely, A.K., Gillespie, A.T., 1992. Review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). Ann. Appl. Biol. 121, 431–454.

Moorhouse, E.R., Easterbrook, M.A., Gillespie, A.T., Charnley, A.K., 1993a. Control of *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae) larvae on a range of hardy ornamental nursery stock species using the entomogenous fungus *Metarhizium anisopliae*. Biocontrol Sci. Technol. 1, 63–72.

Moorhouse, E.R., Gillespie, A.T., Charnley, A.K., 1993b. Application of *Metarhizium anisopliae* (Metsch.) Sor. conidia to control *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) larvae on glasshouse pot plants. Ann. Appl. Biol. 122, 623–636.

Nelson, E.B., Burpee, L.L., Lawton, M.B., 1994. Biological control of turfgrass diseases. In: Leslie, A. (Ed.), Handbook of Integrated Pest Management for Turf and Ornamentals. CRC Press, Boca Raton, FL, pp. 409–427.

O'Connell, K.P., Goodman, R.M., Handelsman, J., 1996. Engineering the rhizosphere: Expressing a bias. Trends Biotechnol. 14, 83–88.

Poprawski, T.J., Marchal, M., Robert, P.-H., 1985. Comparative susceptibility of *Otiorhynchus sulcatus* and *Sitona lineatus* (Coleoptera: Curculionidae) early stages to five entomopathogenic Hyphomycetes. Environ. Entomol. 14, 247–253.

Raid, R.N., Cherry, R.H., 1992. Effect of soil parameters on pathogenicity of the fungus *Metarhizium anisopliae* to the sugarcane grub *Ligrus subtropicus* (Coleoptera: Scarabaeidae). Fla. Entomol. 23, 179–184.

Rath, A.C., 1988. Developments in the use of *Metarhizium anisopliae* for control of subterranean red-headed cockchafer (Coleoptera: Scarabaeidae: *Adoryphorus couloni*). In: Stahle, P.P. (Ed.), Proceedings of the 5th Austral. Conf. Invert. Ecol. Melbourne University. D&D Printing, Victoria, pp. 88–95.

Samuels, K.D.Z., Pinnock, D.E., Bull, R.M., 1990. Scarabaeid larvae control in sugarcane using *Metarhizium anisopliae*. J. Invertebr. Pathol. 55, 135–137.

SAS Institute., 1999. The SAS Statistical System, version 8. SAS Institute, Cary, NC..

Schmidt, E.L., 1979. Initiation of plant root–microbe interactions. Ann. Rev. Microbiol. 33, 355–376.

Schroth, M.N., Hancock, J.G., 1982. Disease-suppressive soil and root-colonizing bacteria. Science 216, 1376–1381.

Soares Jr., G.G., Marchal, M., Ferron, P., 1983. Susceptibility of *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) larvae to *Metarhizium anisopliae* and *Metarhizium flavoviride* (Deuteromycotina: Hyphomycetes) at two different temperatures. Environ. Entomol. 12, 1886–1890.

St. Leger, R.J., Staples, R.C., Roberts, D.W., 1993. Entomopathogenic isolates of *Metarhizium anisopliae*, *Beauveria bassiana*, and *Aspergillus flavus* produce multiple extracellular chitinase isozymes. J. Invertebr. Pathol. 61, 81–84.

St. Leger, R.J., Joshi, L., Bidochka, M.J., Rizzo, N.W., Roberts, D.W., 1996. Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoviride*, and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Appl. Environ. Microbiol. 62, 907–912.

Storey, G.K., Gardner, W.A., 1987. Vertical movement of commercially formulated *Beauveria bassiana* conidia through four Georgia soil types. Environ. Entomol. 16, 178–181.

Storey, G.K., Gardner, W.A., 1988. Movement of aqueous spray of *Beauveria bassiana* into the profile of four Georgia soils. Environ. Entomol. 17, 135–139.

Storey, G.K., Gardner, W.A., Tollner, E.W., 1989. Penetration and persistence of commercially formulated *Beauveria bassiana* conidia in soil of two different tillage systems. Environ. Entomol. 18, 835–839.

Veen, K.H., Ferron, P., 1966. A selective medium for the isolation of *Beauveria tenella* and of *Metarhizium anisopliae*.. J. Invertebr. Pathol. 8, 268–269.

Villani, M.G., Krueger, S.R., Schroeder, P.C., Consolie, F., Consolie, N.H., Preston-Wilsey, L.M., Roberts, D.W., 1994. Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. Environ. Entomol. 23, 502–513.